
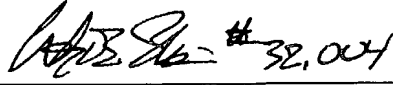


JC07 Rec'd PCT/EP 10 05 MAR 2002

FORM PTO-1390 (REV 10-95)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371				SCH 1868
				U.S. APPLICATION NO. (If known, see 37 CFR §1.5)
INTERNATIONAL APPLICATION NO PCT/EP00/08693		INTERNATIONAL FILING DATE 6 SEPTEMBER 2000		PRIORITY DATE CLAIMED 8 SEPTEMBER 1999
TITLE OF INVENTION L-SELECTIN CONTRAST AGENTS				
APPLICANT(S) FOR DO/EO/US DEBUS, Nils-Peter, et al				
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) <ol style="list-style-type: none"> <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)) <ol style="list-style-type: none"> <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau <input type="checkbox"/> have not been made, however, the time limit for making such amendments has NOT expired. <input checked="" type="checkbox"/> have not been made and will not be made <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)). <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)). <p>Items 11. to 16. below concern document(s) or information included:</p> <ol style="list-style-type: none"> <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98 <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input type="checkbox"/> A change of power of attorney and/or address letter. <input type="checkbox"/> Other items or information: 				

U.S. APPLICATION NO (if known, see 37 CFR §1.5) 10/070304		INTERNATIONAL APPLICATION NO PCT/EP00/08693		ATTORNEY'S DOCKET NUMBER SCH 1868	
17. <input checked="" type="checkbox"/> The following fees are submitted. BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)): Search Report has been prepared by the EPO or JPO..... \$890.00 International preliminary examination fee paid to USPTO (37 CFR §1.482)..... \$710.00 No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2)).. \$740.00 Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO..... \$1040.00 International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 C.F.R. §1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	3	x \$ 18.00	\$54.00	
Independent claims	5 - 3 =	2	x \$ 84.00	\$168.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$ 280.00		
TOTAL OF ABOVE CALCULATIONS =				\$1,112.00	
Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be					
SUBTOTAL =				\$1,112.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 C.F.R. §1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
TOTAL NATIONAL FEE =				\$1,112.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). \$40.00 per property.					
TOTAL FEES ENCLOSED =				\$1,112.00	
				Amount to be refunded:	
				charged:	
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$1,112.00</u> to cover the above fees is enclosed b. <input type="checkbox"/> Please charge my Deposit Account No. <u>13-3402</u> in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>13-3402</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO Customer Number 23,599 PATENT TRADEMARK OFFICE					
 23599			 SIGNATURE Anthony J. Zelano NAME <u>27,969</u> REGISTRATION NUMBER		
Filed: 5 MARCH 2002 AJZ:kmo					

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No. : PCT/EP00/08693
 International Filing Date : 6 SEPTEMBER 2000
 Priority Date(s) Claimed : 8 SEPTEMBER 1999
 Applicant(s) (DO/EO/US) : DEBUS, Nils-Peter, et al

Title: L-SELECTIN CONTRAST AGENTS

PRELIMINARY AMENDMENT

Commissioner for Patents
 Washington, D.C. 20231

SIR:

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

IN THE CLAIMS:

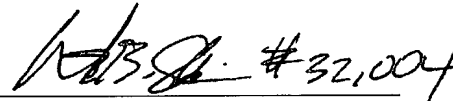
17. (Amended) Contrast media according to claim 1, wherein the receptor is coupled with the aid of a coupling group to the signal unit.

REMARKS

The purpose of this Preliminary Amendment is to eliminate multiple dependent claims in order to avoid the additional fee. Applicants reserve the right to reintroduce claims to canceled combined subject matter.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned "**Version With Markings to Show Changes Made**".

Respectfully submitted,

 #32,004

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Filed: 5 March 2002

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claim 17 was amended as follows:

17. (Amended) Contrast media according to ~~one of~~ claims 1 to 16, wherein the receptor is coupled with the aid of a coupling group to the signal unit.

WO 01/17566

PCT/EP00/08693

L-Selectin Contrast Media

The invention relates to the field of contrast media for imaging diagnosis and describes new contrast media for visualization of lymph node changes and inflammatory processes as well as pathological changes that are associated with the specific expression of endothelial and/or leukocytic ligands.

Endothelial and leukocytic ligands are defined below as the ligands in lymph nodes Sgp50/GlyCAM-1 (Imai et al., 1993), CD34 (Baumheter et al., 1993), MadCAM-1 (Berg et al., 1993), Sgp200 (Hemmerich et al., 1994), the ligands in leukocytes PSGL-1 (Moore et al., 1992) and KG1a ligand (Sackstein et al., 1997), as well as the L-selectin ligands in inflamed vascular endothelia (Zakrzewicz et al., 1997; Girard and Springer, 1995).

As imaging processes, nuclear spin tomography, ultrasonic imaging, imaging with use of x rays (x-ray or computer tomography), scintigraphic imaging with use of radionuclides (gamma camera imaging, SPECT, PET), and imaging by means of near-infrared radiation (NIR radiation) are used.

Up until now, lymph node changes have been diagnosed by means of imaging with use of x rays (computer tomography) based on volume increase or are visualized in nuclear spin tomography by administration of contrast media that are taken up non-specifically by macrophage populations of the lymph node (magnetites or high-molecular gadolinium-containing contrast media for magnetic resonance imaging).

Up until now, inflammatory processes have been visualized non-specifically by the detection, either directly or with assisting contrast medium administration, of the increased discharge of plasma liquid into the inflamed tissue. As an alternative, such processes can also be diagnosed by leukocyte populations, which were labeled in vitro or in vivo beforehand with suitable signal molecules. As signal molecules, in this case, specific radionuclide-labeled antibodies are used against leukocyte surface antigens or magnetites that were phagocytized in vitro by certain leukocyte populations.

All of these processes attempt to visualize lymph node changes and inflammatory processes indirectly.

At present, there exists no demonstrably functioning direct and specific detection of changes in lymph nodes and inflammatory processes as well as pathological changes that are associated with the specific expression of endothelial ligands, by means of imaging diagnosis.

International Patent Application WO 93/06835 describes an adhesion molecule for leukocytes, so-called L-Selectin, and provides details on its use for the visualization of inflammatory processes. In particular, the structure of the cDNA-nucleotide sequence of this molecule and the corresponding amino acid sequence is disclosed. It is proposed to use this protein as a contrast medium for visualizing inflammatory processes by the protein being labeled with radionuclides or paramagnetic substances. A detailed description of the production of such contrast media is lacking, however. There are no details on how

the labeling of the proteins is to take place, and also no examples that the labeled proteins actually show in vivo the desired effects.

As before, there is a great need for specific contrast media for the visualization of lymph node changes or inflammatory processes as well as a need for specific media for visualizing pathological changes, which are associated with the specific expression of endothelial ligands.

The object of this invention is therefore to provide these specific contrast media as well as process for their production.

This object is achieved with the new contrast media and the process for their production.

The new contrast media contain a receptor, e.g., adhesion molecules, such as the L-selectin molecule, in its biologically correct spatial orientation, so that the contrast medium can bind specifically and selectively to endothelial ligands. Biologically correct orientation means that the N-terminal protein end with the ligand-bindable lectin domain points outward away from the cell or from a vehicle or the signal unit, while the C-terminal protein end of the complete protein sequence or a shortened protein sequence points into the cell or inward toward the cell or toward a vehicle or toward the signal unit. The term receptor is defined as a molecular unit, which binds specifically to the above-defined endothelial and leukocytic ligands.

The finding that the contrast medium then binds especially well to its target structure if the receptor is present as a multimer was especially surprising. This means that the contrast

medium then preferably binds to the endothelial ligands, if at least two adhesion molecules, e.g., two L-selectin molecules, are defined and are coupled pointing at a signal unit.

The invention therefore relates to contrast media for visualizing lymph node changes, inflammatory processes or pathological changes, which are associated with the specific expression of endothelial ligands and which are characterized in that a receptor, a receptor fragment or a group of receptors for specifically expressed endothelial and/or leukocytic ligands are coupled in a defined orientation to a signal unit. The receptor is preferably an L-selectin molecule, an L-selectin derivative or a fragment of L-selectin. The signal units vary depending on the application: for nuclear spin tomography, the signal unit must consist of paramagnetic or superparamagnetic particles; superparamagnetic iron oxide particles are preferred. For this application, gadolinium complexes or other paramagnetic metal complexes can also be used. For imaging by means of x rays, iodine-containing molecules or those with heavy metal atoms are preferably used. For ultrasonic imaging, stabilized gas bubbles are used. In radiodiagnosis, radionuclides are used as a signal unit. Finally, near-infrared dyes can be used as signal units for contrast media in near-infrared diagnosis.

The multimerization of the receptor can be carried out in two different ways. First, several receptors (e.g., L-selectin) can be defined and can be coupled pointing at a signal unit. Second, multimerized receptors (e.g., multimeric L-selectin) can already be coupled to the signal unit.

a) Directed Coupling of the Receptor to the Signal Unit

To make possible a directed coupling of monomeric L-selectins to particulate vehicles, protein forms were made that are provided on the C-terminus with a coupling group to ensure the correct spatial orientation (Example 2). A coupling structure, which optimally meets the requirements of high affinity and small size, is the polyhistidine tag (multi-His, e.g., that consists of 4 or more histidine molecules), which interacts with Ni- or Co-complexed chelating agents (simplified nickel chelating agents are mentioned below).

The directed coupling can be carried out by a multi-His-tagged selectin being bonded to a suitable chelating agent via, e.g., Ni^{2+} ions. For this purpose, the chelating agents are coupled to the signal unit. If the signal unit is a ferrimagnetic nanoparticle, such as, e.g., a dextran magnetite, the coupling is carried out by oxidation with sodium periodate. The coupling chemistry is based on the oxidation of diol groups, which are present in carboxydextran. Since such diol groups are also present in starch, those ferrimagnetic nanoparticles are also suitable whose shell materials consist of starch, carboxydextran or comparable materials. In addition, the chelating agents can be coupled to non-radioactive and radioactive colloid particles that are coated with dextran or comparable shell materials. The chelating agents can also be coupled to compact or filled surface-modified polymer spheres. The filling of the polymer spheres can consist of a gas, an x-ray-opaque material, a radioactive substance or fluorescent

dyes, such as, e.g., NIR-dyes. The chelating agents can also be coupled to dendrimer structures, which in turn are doped with fluorescent dyes; x-ray-opaque, paramagnetic, ferrimagnetic signal units or with gas-filled surface-modified polymeric signal molecules.

The chelating agents that are coupled to particulate vehicles (nanoparticles, colloid particles, polymer spheres), which make possible the binding to the multi-His-modified receptors, can contain radioactive metals or metal ions, which emit a signal in diagnostic imaging.

The directed coupling of selectins can be achieved in addition by other molecular properties. Thus, for example, the signal units can carry streptavidin or other avidin variants on their surface or in their structure and bind biotinylated selectins and vice versa. A selectin-specific antibody can also be coupled to the signal units, which binds outside of the active center of the selectin and thus does not destroy its bonding ability after coupling to the signal particles. Another process for the production of contrast media according to the invention therefore consists in that the C-terminus of an L-selectin molecule is coupled to a streptavidin, avidin or biotin molecule, the signal unit contains a biotin, streptavidin or avidin molecule, and the coupling is produced by the specific bond between streptavidin and biotin or avidin and biotin when the L-selectin molecules are combined with the signal unit.

b) Coupling with the Aid of Multimerized Receptors

A second way of coupling L-selectin molecules pointed at a signal unit is to use L-selectin chimera (Example 3). L-Selectin-IG chimera consist of L-selectin and immunoglobulin domains. In this case, the type of immunoglobulin fragment (Ig) that is used determines the degree of multimerization of the chimera: Ig of the γ -class carry 2 Fab fragments, which were exchanged for L-selectin. Thus, L-selectin-IgG chimera carry 2 L-selectin fragments per molecule.

Ig of the μ -class carry up to 10 Fab fragments, which were exchanged for L-selectin. The L-selectin-IgM chimera consequently carry up to 10 L-selectin fragments per molecule. The distance of the ligand binding centers of the L-selectins in the chimera subunits corresponds to the original distance of the Fab fragments and is in general between 1 and 8 nm, in most cases about 5 nm.

Chimera molecules can also be formed from L-selectin and other multimerizing proteins. The mannose-binding protein, consisting of 4 subunits, thus can be doped with 4 L-selectin units. The cartilage oligomeric matrix protein (COMP, Tomschy et al., 1996) in turn consists of 5 subunits, which can be linked by N-terminal with the C-terminal end of the L-selectin.

The chimera molecules that consist of selectin and the Fc portion of antibodies can be bonded either via an antibody against the Fc portion or via protein A, protein L or protein G -- bacterial cell wall molecules, which bind to the Fc portion of immunoglobulins -- pointing (and already multimerized) toward

particle surfaces. The coupling of protein G to the surface of dextran magnetites is described in Example 14.

All above-mentioned chimera molecules that consist of selectin and a multimerization portion can have further modifications on the C-terminal end, e.g., a multi-His tag (Example 4) to which in turn chelating agents can bind, which are coupled in turn to signal units, as described in Example 5.

Various signal units are associated with the receptor or the receptor groups, depending on in which diagnostic process the contrast medium is to be used.

For example, as signal units, ferrimagnetic particles (magnetites, ferrites, cobalt ferrites, i.a.) can be used, which consist of a monocrystalline ferrimagnetic core and a shell (Examples 13 and 14). The shell is connected covalently to the core or encloses the core completely without a direct chemical bond. The shell can consist of dextran, starch or low- or high-molecular aliphatic or aromatic chains. The shell either directly has at its disposal functional groups (amino, carboxyl, thiol groups, i.a.), which can be used for further coupling, or groups that are functionalized after chemical activation for further coupling. Such compounds are described in, e.g., WO 92/12735, WO 92/22586, EP 0 186 616 and US 4,101,435.

Compounds can also be used that consist of the combination of ferrimagnetic particles and compounds that are covalently coupled thereto, whereby the covalently coupled compounds can be provided with functional groups or can contain longer-chain dopable aliphatic compounds.

As signal units, paramagnetic metals and metal compounds can also be used (especially gadolinium complexes). In this case, the metal atom is complexed by a chelating agent that has further functional groups (amino, carboxyl, thiol groups, i.a.) that are mediated, moreover, directly or via a vehicle and that can be used for further coupling. In this case, the chelating agent can also be doped with dendrimers (Example 15), as they are described in, e.g., DE 43 444 60.

The above-mentioned requirements are also met by compounds that consist of the combination of chelating agent and another element, which for its part has functional groups that can be used for further coupling. According to the invention, dopable dendrimers, dopable longer-chain aliphatic compounds, or dopable particles with a diameter of 4-200 nm, consisting of magnetites, polystyrene, dextran, starch, etc., can be used as such elements.

As signal units, x-ray-opaque molecules (e.g., iodine compounds), metals, metal compounds and colloids (e.g., colloidal gold particles, see Example 6, 7 or 12) can be used. In this connection, the receptors or receptor groups are associated directly or indirectly with the x-ray-opaque substances analogously to the above-mentioned. Thus, iodine-containing compounds with coupling groups are used, to which chelating agents for binding multi-His-L-selectrins or Fc-binding substances for binding L-selectin chimera were coupled. Indirectly connected means that the x-ray-opaque molecules are stored in the signal units, whereby the latter are directly connected to the receptors or receptor groups.

As signal units, radioactive molecules, metals, metal compounds and colloids (e.g., colloidal ^{198}Au particles or ^{199}Au particles, see Examples 10 and 11), which are bonded to the receptors or receptor groups analogously to the above-mentioned, can be used.

The binding of L-selectin to its ligands is carried out preferably under the influence of shearing force (Finger et al., 1996). If particulate signal units are coupled to L-selectin, additional shearing forces are induced that result in an improved binding of the L-selectin-particle construct in comparison to pure L-selectin (see Example 8).

As signal units, fluorescent dyes (e.g., NIR dyes) can be used. These can be coupled either directly to the receptors or receptor groups analogously to the above-mentioned or indirectly to the latter (Examples 18 and 19). The dyes in the dendrimers thus can be doped, bonded in compact or hollow dye-containing polymer particles or coupled directly to substances (protein A, protein L, protein G or specific antibodies directed against the multimerization domains), which in turn bind to the multimerization domains of chimera L-selectin molecules. Suitable dye molecules and production thereof are described in, e.g., WO 96/17628.

As signal units, gas-filled, surface-modified polymer spheres can be used, which are coupled to the receptors or receptor groups analogously to the above-mentioned (see Examples 20 to 22).

Examples

The following examples explain the invention.

Example 1: Cloning and Expression of L-Selectin

A clone that contains the entire coding area of L-selectin and is flanked by 5'- and 3'-non-translated areas was isolated from the Igt10 cDNA bank of the human lymphoma cell line Raji. The latter was integrated in the pCRII vector of the TA-Cloning System^(R). The construct resulting therefrom was referred to as LamTA4 and formed the basis for the production of L-selectin constructs. This produces the abbreviation for naming the L-selectin construct from the cloned sequence section (see also International Patent Application WO 93/06835). "sL" stands for "soluble" L-selectin, which corresponds to the soluble form of the molecule, as it occurs in human serum. This form contains the lectin domains, and the EGF and 2 SCR domains. The latter lacks, however, the cytoplasmatic domain and the transmembrane domain, whereby in the construction, the physiological interface, in which sL-selectin is separated from the cell surface proteolytically, is maintained. The sL fragment was generated by means of PCR from starting clone LamTA4 and is integrated again in vector pCRII of the TA-Cloning System^(R). The recombinant sL-selectin was produced as follows in a glycosylated form. The sL-selectin sequence section was cloned in vector pCR3.1 of the TA cloning System^(R) for the expression in K562 cells and in vector pMPSV-HE for the expression in BHK cells.

Then, clones were isolated and selected by means of genitacin (in the case of the pCR3.I vector) or after co-transfection with puromycin-resistance-mediating vectors (in the case of the pMPSV-HE vector). The culture supernatants of L-selectin-secreting K562 or BHK cells were purified by means of immunoaffinity chromatography on CNBr-activated sepharose matrix, to which the antibody DREG200 (Kishimoto et al., 1990) was bonded. The quantification of the amounts of L-selectin to determine the yield was carried out by means of ELISA with use of the antibodies DREG200 and DREG55 (Kishimoto et al., 1990).

Example 2: Cloning and Expression of Multi-His-L-Selectin

With use of a partially complementary PCR primer, which in addition carries the sequence of six histidines (multi-His), an L-selectin construct with a C-terminal coupling group was generated starting from the original clone LamTA4 (see Example 1). The sL-selectin-multi-His fragment (codes the protein multi-His-L-selectin) was cloned in expression vectors pCR 3.1 (invitrogen) and with consideration of the reading grid in SRA-GS-Seq (Berlex Lab., Inc.) and the Baculo Transfer Vector pBBS 250 (Berlex Lab., Inc.). BHK cells were transfected with a multi-His-sL-selectin construct with the addition of a genitacin-resistance-mediating vector. Clones were isolated that secrete the protein multi-His-L-selectin in the culture supernatants.

The multi-His-sL-selectin construct in the Baculo transfer vector was converted to the expression of multi-His-L-selectin in insect cells and then transferred into the viral genome. With

these recombinant viruses, Sf9, High FiveTM and Estigmene acrea cells were infected. The recombinant proteins were, as described in Example 1, purified by means of immunoaffinity chromatography on DREG200.

Example 3: Production and Expression of L-Selectin-Ig Chimera

The L-selectin-IgG chimera, which carry 2 L-selectins per molecule, were produced starting from the following constructs: Mouse-L-selectin-human-IgG chimera (hIgG-mLS) in the pCMV5 expression vector (Watson et al., 1990), rat-L-selectin-human-IgG chimera (hIgG-rLS) in the pCDM8-expression vector (Tamatani et al., 1993), human-L-selectin-human-IgG chimera (hIgG-hLS) in the pCMV5-expression vector (Mebius and Watson, 1993).

Immunoglobulins of the μ -class carry 5 μ -globulin molecules that are arranged in the shape of a star and thus carry 10 Fab fragments that have been exchanged for L-selectin. L-Selectin-IgM chimera thus carry 10 L-selectin fragments per molecule. Starting from a construct in the pCDM8-expression vector, the mouse-L-selectin-human-IgM chimera was produced (Maly et al., 1996).

The recombinant expression vectors were amplified in *E. coli*, purified and then transfected in eukaryotic cells.

The recombinant vectors of the mouse-L-selectin-human-IgM chimera were co-transfected in COS-7 and in CHO-K1 cells with a genitacin-resistance-mediating vector. Stably producing clones of both cell lines were isolated. In addition, the cDNA fragment, which codes for the chimera protein, was cut out with

restriction endonucleases from the vector pCMD8 and subcloned in the vector pcDNA3. With this construct, HEK293 cells were transfected and clones were isolated. The purification of the hlgM-mLS chimeras was carried out by means of immunoaffinity chromatography on MEL-14 (Bowen et al., 1990). The production and purification of chimeras was monitored by means of ELISAs (MEL-14 as a capturing antibody and anti-hlgG-phosphatase as a detection antibody).

The IgG chimera (human, rat and mouse-L-selectin-human-IgG chimera) were produced by co-transfection with vector pcDNA3 in stably producing HEK293 and CHO-K1 cells. The purification of the hlgG-LS chimeras was carried out by means of affinity chromatography on protein G (Pharmacia): After the culture supernatant was applied on the column, the matrix was washed with 20 mmol of sodium phosphate (pH 7.0). It was eluted with 0.1 M glycine (pH 2.5), and the eluates were neutralized with saturated K_2HPO_4 solution.

Example 4: Production and Expression of L-Selectin-Ig-Multi-His-Chimera

The mouse-L-selectin-human-IgG chimera (hlgG-mLS) in the pCMV5-expression vector (see Example 3) was extended by a C-terminal histidine coupling group that attaches to the Fc fragment of the chimera with use of a partially complementary PCR primer, which in addition carries the sequence of six histidines (multi-His) (see Example 2). The amplification, purification and transfection of the recombinant expression vector as well as the

isolation of stably producing HEK293 cell clones was carried out just like the production and purification of the multi-His chimera analogously to the procedure described in Example 3.

Example 5: Synthesis of Radioactive Chelating Agent-Signal Units and Coupling of Multi-His-L-Selectins as Scintigraphic Contrast Media

I.a., calcein and Newport Green (Molecular Probes, Inc.) are nickel ion-complexing chelating agents that can bind to multi-His proteins. Moreover, calcein and Newport Green can be iodized and can thus be used as signal units for scintigraphic imaging. With 1 nmol of Newport Green, 10 μ l of 0.25 M phosphate buffer, pH 6.5-7.5 (iodizing buffer) which contained 0.2 mCi of Na^{123}I as well as 5 μ l of chloramine T (8 mg/ml in iodizing buffer) were incubated for 2 minutes at room temperature. The reaction was completed by adding 10 μ l of $\text{Na}_2\text{S}_2\text{O}_5$ (8 mg/ml in iodizing buffer) as well as 100 μ l of NaI (2 mg/ml in iodizing buffer). Then, 1 nmol of L-selectin-IgG-multi-His chimera (produced according to Example 4) and 1 μ l of 50 mmol of Ni acetate (50 nmol) were added to the iodized Newport Green. After 20 minutes of incubation at room temperature, the product (L-selectin-IgG-multi-His chimera)-(Ni)-(Newport Green)-(^{123}I) was purified on a PD10 column in phosphate buffer from the low-molecular substances (nickel ions, unbonded Newport Green as well as iodizing reactives). Preserving the bonding ability of the iodized l-selectin chimera was detected in cryosections with subsequent microautoradiography.

Example 6: Synthesis of Colloidal Gold

For a 100 ml batch, 80 ml of distilled water and 50 μ l of 20% HAuCl_4 were introduced and heated to 60°C. After a solution that contains 4 ml of 1% Na citrate, 18 ml of distilled water and 80 μ l of 1% tannin was added, the reaction mixture was heated to 95°C for 10 minutes. The changing of the solution's color from yellow to red shows the formation of colloid particles. The product was cooled and sterilized by filtration. The diameter of the colloids was 8-12 nm.

Example 7: Synthesis of L-Selectin-Ig-Chimera-Protein G-Colloidal Gold Constructs

300 μ l of protein G (2 mg/ml in distilled water) was added for 1 hour at room temperature to 10 ml of colloidal gold (for production, see Example 6). Free unbonded protein G that is present in the solution was separated by repeated washing from buffer (10 mmol of HEPES, pH 7.4, 150 mmol of NaCl) by means of centrifuging at 30,000 g with a Sorvall 80AT3 rotor. The protein G-gold colloids were taken up in 300 μ l of buffer. By tracer measurements, it was possible to show that about 15 protein G-molecules were bonded to a 10 nm colloidal gold particle. Then, the IgG-selectin chimeras were coupled to these constructs. 50 μ g of mouse or rat-L-selectin-IgG chimera were added to 300 μ l each of protein G-colloidal gold. After 1 hour of incubation at room temperature, about 80-90% of the amount of chimera used could be bonded to the protein G-gold colloids. The bonding ability of the IgG-selectin chimera-protein G-gold colloid

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Example 8: Surface Plasmon Resonance (SPR) Measurements with L-Selectin-Ig-Chimera-Protein G-Colloidal Gold Constructs

Mouse- or rat-L-selectin chimera-protein G-gold colloid constructs (for production, see Example 7) were diluted one hundred times with BIAcore running buffer HBS+Ca²⁺ (10 mmol of HEPES, pH 7.4; 150 mmol of NaCl; 0.005% P20; 1 mmol of CaCl₂). As a negative control, the constructs were diluted in HBS without the addition of Ca²⁺. Then, the substances were added via an SA-Pioneer Chip (BIAcore 2000/BIAcore AB, Freiburg) at a flow rate of 20 μ l/minute. The flow cells were previously charged with 100 RU of multivalent polyacrylamide-biotin-derivatized ligands in each case (Syntesome GmbH, Munich): Fc-1 = N-acetyllactosamine (negative control), Fc-2 = sialyl-Lewis^x (L-selectin ligand), Fc-3 = sulfo-tyrosine-sialyl-Lewis^x (optimized L-selectin ligand). The binding of the L-selectin chimera-gold colloid constructs was carried out exclusively in the presence of calcium ions on Fc-2 with 250 RU, on Fc-3 with 700 RU. Without the addition of calcium or with use of pure L-selectin chimera (without gold colloid concentration), no bond or measuring signal was detected in the range of 1 RU.

Example 9: Active Detection with L-Selectin-Ig-Chimera-Protein G-Gold Colloid Constructs

NMRI mice: Each animal was intravenously injected with 12 μ g of mouse- or rat-L-selectin-chimera protein G-gold colloid constructs (for production see Example 7; amount of substance relative to the L-selection chimera portion) or, as a control, uncharged protein G-gold colloid constructs. At various times, the peripheral lymph nodes were removed and analyzed in frozen sections with subsequent silver staining. Immediately after the "first pass" up to a period of 30 minutes after injection, gold colloids on the luminal side of the high-endothelial venules in the lymph nodes could be detected in the case of animals treated with selectin chimera constructs. Animals that obtained the control substance did not show any gold colloids whatsoever in the lymph nodes.

Example 10: Synthesis of ^{198}Au -Colloid Protein G-L-Selectin-Ig-Chimera Constructs (as Scintigraphic Contrast Media)

100 μ g of the L-selectin-Ig-chimera protein G-gold colloid constructs (for production, see Example 7) was irradiated in the neutron ray activation unit of the nuclear reactor BERII in distilled water until a specific activity of 300-500 MBq/100 μ g relative to the L-selectin portion was achieved.

Example 11: In-Vivo-Scintigraphic Measurement after Administration of ^{123}I -Newport Green-L-Selectin Contrast Media or ^{198}Au -Colloid-Protein G-L-Selectin-Ig-Chimera Contrast Media

The rabbits were anesthetized with ketamine/xylazine. The constructs according to Example 10 were intravenously injected with a specific activity of 300-500 MBq/100 μg relative to the L-selectin portion. By means of dynamic measurement on a minute cycle and ultimately with a static measurement after 30 minutes, the animals were examined with the gamma camera SP4HR (Elscint, now General Electric). In this case, primarily the lymph nodes were studied. The animals were positioned under the camera, and the first study was carried out before the contrast medium was administered. Subsequent to the injection, the animal was examined at various above-mentioned times. In this case, an APC 3-collimator was used for the imaging with ^{123}I iodine isotopes, and an APC 6-collimator was used for the visualization of the ^{198}Au isotopes. Position, size and appearance of the individual lymph nodes were determined.

Example 12: In-Vivo-X-Ray-Imaging after L-Selectin-Ig-Chimera-Protein G-Gold Colloid Contrast Media are Administered

The animals (mice) were anesthetized i.p. with 0.05 ml of ketamine/Rompun (2:1). In each case, 25 μg of the L-selectin-Ig-chimera-protein G-gold colloids (produced according to Example 7) or protein G-gold colloids (as a negative control) were injected intravenously. After 30 minutes, the animals were examined with a 7x zooming mammography (Picker), and the cervical and popliteal

lymph nodes were visualized at 25 kV and 10 mAs irradiation. The visualization of the contrast medium concentration in the lymph nodes was carried out at 45 kV and 4.5 mAs irradiation.

Example 13: Synthesis of Particulate Chelating Agent-Magnetites and Coupling of Multi-His-L-Selectin as a Magnetic Resonance Contrast Medium

Coupling of NTA (Nitrilotriacetic acid derivative; α -N-[bis-carboxymethyl-]lysine) to dextran magnetites

The dextran magnetites (US 4,101,435) were oxidized in aqueous solution with a 31x partial excess of sodium periodate (relative to the carboxydextran of the dextran magnetite shell) for 30 minutes while being stirred in the dark at room temperature. Then, the sodium periodate was separated quantitatively via a gel filtration. The dextran magnetites were eluted in phosphate buffer (0.1 M phosphate buffer, pH 7.0). Then, NTA was added to the oxidized dextran magnetites and incubated for 2 hours at room temperature while being shaken intermittently in the dark. In this case, it was possible to couple NTA in excess to the dextran magnetites. Then, 1/10 volume of the reducing agent dimethylborane (150 mmol in H₂O) was added and incubated for another 2 hours at room temperature while being shaken intermittently in the dark. The last step was repeated, followed by an incubation at 4°C overnight. The separation of the unbonded NTA from NTA that is bonded to the surface of the dextran magnetites was carried out via gel filtration or ultrafiltration. The dextran magnetites were eluted in PBS or in

0.1 M HEPES (in each case pH 7.0-7.4) and stabilized by the addition of 5 mg/ml of carboxydextran (final concentration). The particles were sterilized by filtration, and sodium azide was added in a final concentration of 0.1%. Then, the iron content of the suspension as well as the mean particle size of the particles were determined.

To examine the coupling efficiency of the NTA on the particle surface, the dextran magnetites were first incubated with 10 mmol of EDTA in PBS or 0.1 M HEPES for 1 hour at room temperature while being shaken intermittently. Then, the EDTA was separated via gel filtration or ultrafiltration, and the sample was incubated with Co^{2+} , Ni^{2+} or comparable divalent ions, which are complexed by the chelating agent. Excess ions were then separated via gel filtration or ultrafiltration from the particles. It was possible to determine the number of NTA molecules bonded to the particle surface by an ICP measurement of the bonded ions in the case of subtraction of the ions, which bind to unmodified dextran magnetites.

Coupling of Multi-His-L-Selectin to NTA-Dextran Magnetites

The NTA-carrying dextran magnetites were first incubated with Ni^{2+} ions (ions, or the like) and then incubated with multi-His-tagged selectin molecules in PBS or 0.1 M HEPES with 0.2% milk (for reduction of non-specific bonds) for 10 minutes at room temperature. Unbonded selectin molecules were separated via suitable ultrafiltration units or via magnetic columns (Miltenyi Biotec) when a magnetic field is employed). The resulting

contrast medium constructs were examined *in vitro* for their bonding ability, e.g., in the frozen sections of peripheral mouse lymph nodes, and could then be used for *in-vivo* experiments for imaging.

Example 14: Synthesis of Protein G-Magnetites and Coupling of L-Selectin-Ig Chimeras

Coupling of Protein G to Dextran Magnetites

The dextran magnetites were first described as in the above example, oxidized with sodium periodate. Excess sodium periodate was separated via gel filtration, and the dextran magnetites were eluted in sodium acetate buffer (100 mmol of sodium acetate buffer, pH 3.9).

Then, protein G was added and incubated for 2 hours at room temperature in the dark while being shaken intermittently. The reduction with dimethylborane was carried out as described in the example above. Before the incubation, which occurs overnight, 5 mg/ml of carboxydextran (final concentration) was added in addition to stabilize the particles. The separation of the unbonded protein G was carried out via ultrafiltration. The particles were sterilized by filtration, and sodium azide was added in a final concentration of 0.1%. Then, the iron content of the suspension and the particle size of the particles was determined. It was possible to determine the coupling efficiency by the test being performed in the presence of smaller amounts of radiolabeled protein G.

Coupling of L-Selectin-Ig Chimeras

Then, the dextran magnetites that carry protein G were incubated with the IgG-selectin chimera for at least 2 hours at room temperature or overnight at 4°C. The resulting contrast medium constructs were examined *in vitro* for their bonding ability, e.g., in the frozen section of peripheral mouse lymph nodes and could then be used for *in-vivo* experiments for imaging. In the case of sufficient magnetic particles, it was possible to separate in advance the unbonded IgG-selectin chimera via magnetic columns (Miltenyi Biotec) when a magnetic field was employed.

Example 15: Synthesis of Dendrimer-Chelating Agent-Signal Units and Coupling of Multi-His-L-Selectin

Metal chelating agent-carrying dendrimers DSM-64-NTA-Gd-DTPA (for production, see WO 99/32154), which contained up to 28 gadolinium-DTPA complexes as signal units for MR imaging, were used. 10 µg of the dendrimers, with a bonding capacity of up to 30 multi-His proteins per dendrimer, was charged by incubation with 50 mmol of nickel acetate with Ni^{2+} ions for 10 minutes at room temperature and purified via PD10-gel chromatography of unbonded Ni^{2+} ions. Then, the batch was incubated with 7 mg of multi-His-tagged selectin molecules in 10 mmol of HEPES (pH 7.4) for 1 hour at room temperature. Unbonded selectin molecules were separated via gel chromatography by the multi-His-L-selectin dendrimers. The degree of concentration was on average 10-15 multi-His-L-selectins per dendrimer.

The bonding ability of this contrast medium construct was examined in vitro in a frozen section of peripheral mouse lymph nodes, by the complexes after the incubation having been detected on the frozen section with the non-blocking antibody LAM1-14 (Mihelcic et al., 1994) and subsequent staining with a fluorescent secondary antibody.

Example 16: MR Measurement of Ex-Vivo-Agar Phantoms

The L-selectin-Ig-chimera-protein G-magnetite constructs (produced according to Example 14) were intravenously injected in animals (mice, rats, rabbits). At previously set times (e.g., 5 minutes, 0.5 hour, 1 hour), the animals were sacrificed (by injection of an overdose of anesthesia, and various organs (lymph nodes, spleen, liver) were removed and weighed.

Example of in-vivo administration: 4 animals (NMRI, Schering SPF, 18-22 g, female)

Anesthesia: 0.05 ml of ketamine/Rompun (2:1) i.p.

Mouse 1 and 2: Control animals

Mouse 3: Received about 443 ($24.2 \mu\text{g}$) μl of DDM128N protein G i.v. (caudal vein) as a control.

Mouse 4: Received about 525 ($24.3 \mu\text{g}$) μl of DDM128N-protein G-mouse-L-selectin-Ig chimera i.v. (caudal vein).

After 5 minutes, the peripheral lymph nodes (inguinal, iliac, cervical, popliteal, axial), a section of the liver and the spleen were removed and weighed.

Preparation of the agar phantom: Production of an agar phantom: 2% agar solution (for microbiology) with 0.05 mmol of

Magnevist^(R). Agar was heated in the microwave (settings: quick digest: power 70-80%, fan speed 100, time 10 minutes; pressure 10) until the solution was clear and the air bubbles had disappeared. The first layer (about 1-2 cm) was loaded into a rectangular plastic vessel, cooling for about 30-60 minutes at room temperature. The organs were arranged on the agar and covered with a second layer of liquid agar. The cooling was carried out until hardening was completed, then the phantom was stored in a refrigerator.

MR measurement: The phantom was measured with various sequences. To determine position, a T1-weighted sequence was used (e.g., TR 400/TE 15, rare factor = 2, averages = 4, matrix 256 x 256, layer thickness 3 mm).

Then, the phantom was measured by means of iron (magnetite)-sensitive sequences (T2-weighted: TR/TE 2500/14, rare factor = 16, averages = 4. Layer thickness 3 mm, matrix 256 x 256; gradient echo: TR/TE 400/15, averages = 4, 30° flip angle). For the evaluation with an image processing program, ROI's (regions of interest) were put on the individual organs, and the signal intensities were determined and compared.

Example 17: In-Vivo-MR-Measurement after Administration of L-Selectin-Magnetite Constructs

The animals (mice, rats, rabbits) were anesthetized (e.g., ketamine/xylazine), and the constructs were injected intravenously according to Example 14. At previously set times (e.g., 5 minutes, 1 hour, 4 hours), the animals were examined

with magnetic resonance tomography. In this case, primarily the lymph nodes and inflamed areas were studied. The animals were positioned in the MR device, and the first study was carried out before the contrast medium was administered. Following the injection, the animal was examined at the different above-mentioned times with various sequences. To determine the position, a T1-weighted sequence was used (e.g., TR 400/TE 15, rare factor = 2, averages = 4, matrix 256 x 256, layer thickness 3 mm). Then, the animals were visualized by means of iron-sensitive sequences (e.g., T2-weighted: TR/TE 2500/14, rare factor = 16, averages = 4, layer thickness 3 mm, matrix 256 x 256; gradient echo: TR/TE 400/15, averages = 4, 30° flip angle). For the evaluation with an image processing program, ROI's (regions of interest) were put on the individual organs, the signal intensities were determined and compared. Position, size and appearance of the individual lymph nodes were determined.

Example 18: Direct and Indirect Coupling of NIR Dyes to L-Selectin-Ig Chimeras

In a way similar to processes that are known in the literature by reaction with 3-aminopropyl-t-butylcarbamate, and release of the amino group by acidic cleavage with trifluoroacetic acid, 1,1'-bis-(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid is converted into aminopropyl]-bis-1,1'-(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid amide. The latter, referred to below as NIR dye, was covalently coupled directly with the mouse and rat L-selectin-Ig chimeras. The

coupling is carried out via an amino group of the dye to the oxidized chimeras. In this case, the oxidation was carried out by means of sodium periodate with a 31x molecular excess relative to the amount of chimera (100 μ g) for 30 minutes while being stirred in the dark at room temperature. Then, the sodium periodate was separated quantitatively via a gel filtration, and the dye incubation was connected with 50x excess to the NIR dye. Then, 1/10 volume of the reducing agent dimethylborane (150 mmol in H_2O) was added and incubated for another 2 hours at room temperature while being shaken intermittently in the dark. Unbonded dye and reducing agent were separated by ultrafiltration in phosphate buffer (0.1 M phosphate buffer, pH 7.0). The L-selectin-Ig chimeras could be loaded with 2-5 NIR dye molecules each, and their fluorescence quantum yield was about 50% of the starting value. The bonding ability of the NIR-L-selectin-IG chimeras was examined in vitro in the frozen section on peripheral mouse lymph nodes, by the localization of the NIR-fluorescent complexes being shown directly after incubation by means of an NIR-CCD microscope camera.

In the case of indirect labeling, 15 μ g of protein G was oxidized by means of sodium periodate with a 30x molecular excess relative to the amount of protein G -- as described for the direct labeling -- intermediately purified and incubated with 50x excess on NIR dye Li196. Reduction with dimethylborane and subsequent purification are carried out as described above. Then, 10 μ g of the NIR protein G conjugates with equimolar amounts of mouse or rat L-selectin-Ig chimeras (60 μ g) was mixed

Example 19: In-Vivo-NIR Measurement after Administration of L-Selectin-NIR-Constructs

Example 20: Functionalized Gas-Filled Microcapsules

7 l of an aqueous 1% octoxynol solution at a pH of 2.5 is introduced into a 20 l reactor and mixed with a rotor-stator-mixer at a high shear gradient so that a self-gassing with strong foam production is carried out. 100 g of cyanoacrylic acid butyl ester is quickly (< 1 minute) added and dispersed. It is polymerized for 60 minutes under self-gassing, whereby gas-filled

(b) Functionalization of Gas-Filled Microcapsules by Partial Side-Chain Hydrolysis

Example 21: Binding of L-Selectin to Functionalized Gas-Filled Microcapsules

The microcapsule suspension according to Example 20 is purified by flotation at least 5x. 1 ml of the purified microcapsule suspension with a concentration of 5×10^9 particles per ml is rebuffed in 10 mmol of acetate, pH 4.0, and activated with 0.1 M EDC/NHS. Then, it is incubated with 0.25 mg of protein G (5x excess) for one hour at room temperature. The reaction is completed by a 15-minute incubation with 1 M ethanolamine.

The gas-filled microcapsules, to which protein G was bonded, are purified by multiple washing cycles by means of centrifuging at a maximum of 500 g. The purified, gas-filled protein-G-binding microcapsules are incubated overnight with 100 μ g of L-selectin-Ig chimeras.

50% of the amount of L-selectin was bonded to the microcapsules (FACS measurement: saturation series with anti-selectin antibodies).

Example 22: In-Vivo-Sonographic Measurement after Administration of L-Selectin-Ig-Chimera-Protein G-Polymer Microcapsules

The rabbits were anesthetized with ketamine/xylazine. 1 ml of the echogenic L-selectin-Ig-chimera-protein G-polymer microcapsules (L-selectin-polymer microcapsules, see Example 21) with a concentration of 1×10^9 particles/ml in isotonic aqueous dispersion was injected intravenously. As a control, protein G-polymer microcapsules without L-selectin-Ig-chimera concentration were injected. Directly after administration of the contrast medium, the sonographic imaging of the cervical and popliteal lymph nodes in the Harmonic Imaging Modus was begun. Because of the hepatic clearance of unbonded polymer microcapsules, the signal-background ratio of the L-selectin-polymer microcapsules that are accumulating in the target reached optimal values after 30 minutes.

Literature

Baumhater, S.; Singer, M. S.; Henzel, W.; Hemmerich, S.; Renz, M.; Rosen, S. D., and Lasky, L. A. (1993). Binding of L-Selectin to the Vascular Sialomucin CD34, *Science* **262**, 436-8.

Berg, E. L.; McEvoy, L. M.; Berlin, C.; Bargatze, R. F., and Butcher, E. C. (1993). L-Selectin-Mediated Lymphocyte Rolling on MadCAM-1. *Nature* **366**, 695-8.

Bowen, B. R.; Fennie, C., and Lasky, L. A. (1990). The Mel 14 Antibody Binds to the Lectin Domain of the Murine Peripheral Lymph Node Homing Receptor. *J Cell Biol* **110**, 147-53.

Finger, E. B.; Puri, K. D.; Alon, R.; Lawrence, M. B.; von Andrian, U. H.; Springer, T. A. (1996). Adhesion through L-Selectin Requires a Threshold Hydrodynamic Shear. *Nature* **379**, 266-269.

Girard, J. P., and Springer, T. A. (1995). High Endothelial Venules (HEVs): Specialized Endothelium for Lymphocyte Migration. *Immunol Today* **16**, 449-57.

Hemmerich, S.; Butcher, E. C., and Rosen, S. D. (1994). Sulfation-Dependent Recognition of High Endothelial Venules (HEV)-Ligands by L-Selectin and MECA 79, and Adhesion-Blocking Monoclonal Antibody. *J Exp Med* **180**, 2219-26.

Imai, Y.; Lasky, L. A.; and Rosen, S. D. (1993). Sulphation Requirement for GlyCAM-1, An Endothelial Ligand for L-Selectin. *Nature* **361**, 555-7.

Kishimoto, T. K.; Jutila, M. A., and Butcher, E. C. (1990). Identification of a Human Peripheral Lymph Node Homing Receptor: A Rapidly Down-Regulated Adhesion Molecule. *Proc Natl Acad Sci U S A* **87**, 2244-8.

Maly, P.; Thall, A.; Petryniak, B.; Rogers, C. E.; Smith, P. L.; Marks, R. M.; Kelly, R. J.; Gersten, K. M.; Cheng, G.; Saunders, T. L.; Camper, S. A.; Camphausen, R. T.; Sullivan, F. X.; Isogai, Y.; Hindsgaul, O.; von Andrian, U. H.; and Lowe, J. B. (1996). The Alpha(1,3)-fucosyltransferase Fuc-TVII Controls Leukocyte Trafficking through an Essential Role in L-, E- and P-Selectin Ligand Biosynthesis. *Cell* **86**, 643-53.

Mebius, R. E., and Watson, S. R. (1993). L- and E-Selectin Can Recognize the Same Naturally Occurring Ligands on High Endothelial Venules. *J Immunol* **151**, 3252-60.

Mihelcic, D.; Schleiffenbaum, B.; Tedder, T. F.; Sharar, S. R.; Harlan, J. M., and Winn, R. K. (1994). Inhibition of Leukocyte L-Selectin Function with a Monoclonal Antibody Attenuates Reperfusion Injury to the Rabbit Ear. *Blood* **84**, 2322-8.

Moore, K. L.; Stults, N. L.; Diaz, S.; Smith, D. F.; Cummings, R. D.; Varki, A., and McEver, R. P. (1992). Identification of a Specific Glycoprotein Ligand for P-Selectin (CD62) on Myeloid Cells. *J Cell Biol* **118**, 445-56.

Sackstein, R.; Fu, L., and Allen, K. L. (1997). A Hematopoietic Cell L-Selectin Ligand Exhibits Sulfate-Independent Binding Activity. *Blood* **89**, 2773-81.

Tamatani, T.; Kuida, K.; Watanabe, T.; Koike, S., and Miyasaka, M. (1993). Molecular Mechanisms Underlying Lymphocyte Recirculation. III. Characterization of the LECAM-1 (L-Selectin)-Dependent Adhesion Pathway in Rats. *J Immunol* **150**, 1735-45.

Tomschy, A.; Fauser, C.; Landwehr, R.; and Engel, J. (1996). Homophilic Adhesion of E-Cadherin Occurs by a Co-operative Two-Step Interaction of N-Terminal Domains. *Embo J* **15**, 3507-14.

Watson, S. R.; Imai, Y.; Fennie, C.; Geoffroy, J. S.; Rosen, S. D., and Lasky, L. A. (1990). A Homing Receptor-IgG Chimera as a Probe for Adhesive Ligands of Lymph Node High Endothelial Venules. *J Cell Biol* **110**, 2221-9.

Zakrzewicz, A.; Gräfe, M.; Terbeek, D.; Bongrazio, M.; Auch-Schwelk, W.; Walzog, B.; Graf, K.; Fleck, E.; Ley, K.; and Gaehtgens, P. (1997). L-Selectin-Dependent Leukocyte Adhesion to Microvascular but Not to Macrovascular Endothelial Cells of the Human Coronary System. *Blood* **89**, 3228-35.

Claims

1. Contrast media for visualization of lymph node changes, inflammatory processes or pathological changes that are associated with the specific expression of endothelial and/or leukocytic ligands, characterized in that the C-terminal end of a receptor, a receptor fragment or a group of receptors for specifically expressed endothelial ligands is coupled to the signal unit, while the N-terminal end of the signal unit that contains the binding domains is pointed away from the signal unit.

2. Contrast media according to claim 1, wherein the receptor consists of at least 2 molecules.

3. Contrast media according to claim 1, wherein at least 2 molecules show a distance of 1-8 nm at the N-terminal end or else have a distance that corresponds to the distance of the N-terminal ends of chimera molecules, thus, e.g., receptors that were substituted for the Fab fragments of immunoglobulin skeletons.

4. Contrast media according to claim 1, wherein the receptor is an L-selectin derivative.

5. Contrast media according to claim 1, wherein the receptor is L-selectin.

6. Contrast media according to claim 1, wherein the receptor is an L-selectin-Ig chimera.

7. Contrast media according to claim 1, wherein the signal unit contains a paramagnetic particle.

8. Contrast media according to claim 1, wherein the signal unit contains a superparamagnetic particle.

9. Contrast media according to claim 6, wherein the signal unit is a superparamagnetic iron oxide particle.

10. Contrast media according to claim 1, wherein the signal unit is a gas-filled particle.

11. Contrast media according to claim 1, wherein the signal unit contains a paramagnetic metal atom.

12. Contrast media according to claim 1, wherein the signal unit contains a heavy metal ion.

13. Contrast media according to claim 1, wherein the signal unit contains an iodine-containing molecule.

14. Contrast media according to claim 1, wherein the signal unit contains a radionuclide.

15. Contrast media according to claim 1, wherein the signal unit contains a dye molecule, which absorbs near-infrared radiation.

16. Contrast media according to one of claims 1 to 16, wherein the receptor is coupled with the aid of a coupling group to the signal unit.

17. Contrast media according to claim 14, wherein the coupling group is a polyhistidine radical.

18. Process for the production of contrast media for visualization of lymph node changes, inflammatory processes or pathological changes, which are associated with the specific expression of endothelial and/or leukocytic ligands, wherein a

multimerized receptor, which binds to the endothelial ligands, is coupled to a signal unit.

19. Process according to claim 16, wherein the multimerized receptor is an L-selectin-Ig chimera.

20. Process for the production of contrast media for visualization of lymph node changes, inflammatory processes or pathological changes, which are associated with the specific expression of endothelial and/or leukocytic ligands, wherein several receptors that bind to the endothelial ligands are defined and coupled pointing, with the aid of a coupling group, at a signal unit.

21. Process according to claim 18, wherein the coupling group is a polyhistidine radical.

22. Process for the production of contrast media for visualization of lymph node changes, inflammatory processes or pathological changes, which are associated with the specific expression of endothelial and/or leukocytic ligands, wherein the C-terminus of an L-selectin molecule is coupled to a streptavidin, avidin or biotin molecule, the signal unit contains a biotin, streptavidin or avidin molecule, and the coupling is produced by the specific bond between streptavidin and biotin or avidin and biotin when the L-selectin molecules are combined with the signal unit.

23. Use of L-selectin-Ig chimeras for the production of contrast media for the visualization of lymph node changes, inflammatory processes or pathological changes, which are associated with the specific expression of endothelial and/or

leukocytic ligands.



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- Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

WO 01/17566 A2

(54) Title: L-SELECTIN CONTRAST AGENTS

(54) Bezeichnung: L-SELECTIN-KONTRASTMITTEL

(57) Abstract: The invention relates to novel contrast agents for depicting changes in lymph nodes, depicting inflammatory processes, and pathological changes. The inventive contrast agents are bound to the specific expression of endothelial and/or leukocyte ligands. The invention also relates to a method for producing said contrast agents.

(57) Zusammenfassung: Die Erfindung betrifft neue Kontrastmittel zur Darstellung von Lymphknotenveränderungen und entzündlichen Prozessen sowie von pathologischen Veränderungen, die mit der spezifischen Expression von endothelialen und/oder leukozytären Liganden verbunden sind, sowie Verfahren zu deren Herstellung.

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

L-SELECTIN CONTRAST AGENTS

the specification of which

☐ is attached hereto

☐ was filed on 6 September 2000 as United States Application Number or PCT International

Application Number PCT/EP00/08693 and (if applicable) was amended on 6 November 2001

I hereby authorize our attorneys to insert the serial number assigned to this application.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 USC §119			
APPLICATION NO.	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
199 43 710.6	GERMANY	8 SEPTEMBER 1999	YES
100 13 849.7	GERMANY	15 MARCH 2000	YES

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION(S) UNDER 35 U.S.C. §119(e)	
APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under 35 U.S.C. §120 of any United States application, or §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

PRIOR U.S./PCT INTERNATIONAL APPLICATION(S) DESIGNATED FOR BENEFIT UNDER 37 U.S.C. §120		
APPLICATION NO.	FILING DATE	STATUS — PATENTED, PENDING, ABANDONED

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith: I. William Millen (19,544); John L. White (17,746); Anthony J. Zelano (27,969); Alan E. J. Branigan (20,565); John R. Moses (24,983); Harry B. Shubin (32,004); Brion P. Heaney (32,542); Richard J. Traverso (30,595); John A. Sopp (33,103); Richard M. Lebovitz (37,067); John H. Thomas (33,460); Catherine M. Joyce (40,668); Nancy J. Axelrod (44,014); James T. Moore (35,619); James E. Ruland (37,432); Jennifer J. Branigan (40,921) and Robert E. McCarthy (46,044)

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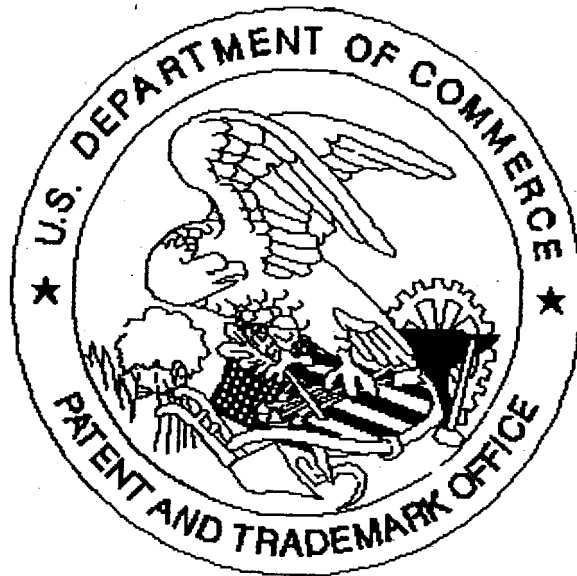
PATENT TRADEMARK OFFICE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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